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Note

Determination of midazolam by high-performance liquid chromatography

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In a previous communication [1] we reported a procedure for the serum determination of midazolam using gas chromatography (GC) with a nitrogen-selective detector. GC with nitrogen-selective detection resulted in a selective procedure for the direct determination of midazolam with a sensitivity of 50 ng/ml. We present, in this paper, a procedure for the direct determination of midazolam and its major metabolite (1-hydroxymethylmidazolam) using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The sensitivity of the assay is increased by the use of HPLC and better linearity was observed in the lower (ng/ml) concentration range.

MATERIALS AND METHODS

For HPLC separations we used a Model 6000A solvent delivery system, Model 600 solvent programmer, Model 440 absorbance detector, Model 450 variable-wavelength detector, Model U6K universal liquid injector (Waters Assoc., Milford, MA, U.S.A.) and a dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.). A prepacked 10- μ m particle size μ Bondapak C₁₈ (300 \times 4 mm I.D.) column from Waters Assoc. was also used.

A Model 5840A gas chromatograph with dual nitrogen–phosphorus sensitive

detectors and coiled glass columns, 1.2 m × 2 mm I.D., packed with 2% SP-2250 or 2% SP-2100 on Chromosorb W HP 100–120 mesh (Hewlett-Packard, Avondale, PA, U.S.A.) were used for GC separations.

Reagents

Heptane and isobutanol were analytical reagent (AR) grade. High-purity methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Sodium hydroxide, 0.05 mol/l, sulfuric acid, 1 mol/l and 0.05 mol/l, were prepared from concentrated solutions. Octanesulfonic acid, 0.05 and 0.005 mol/l, was prepared from its sodium salt, which was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.).

The HPLC mobile phase was octanesulfonic acid, 0.005 mol/l in methanol. Dilute 50 ml of 0.05 mol/l octanesulfonic acid with 150 ml of water and bring the volume to 500 ml with methanol. The pH was adjusted to 3.5 with sulfuric acid.

Standards

Midazolam maleate, flurazepam dihydrochloride and 1-hydroxymethylmidazolam were obtained from Hoffmann-LaRoche (Nutley, NJ, U.S.A.).

Standards of midazolam, its metabolite and the internal standard (flurazepam) were prepared at 1 mg/ml in absolute ethanol. Dilutions of the 1 mg/ml standards were used to make the appropriate working standards of midazolam and its 1-hydroxymethyl metabolite. A 5 mg/l flurazepam solution was also prepared.

Operating conditions

For HPLC we used a C₁₈ reversed-phase column and a mixture of methanol–water (60:40, v/v) as the mobile phase, at a flow-rate of 1 ml/min. The eluent was monitored at 254 and 220 nm with the use of a constant- and variable-wavelength detector.

For GC analysis we used a column temperature of 260°C and a helium flow-rate of 40 ml/min.

Procedure

The extraction procedure has been reported [1]. Midazolam, 1-hydroxymethylmidazolam and flurazepam (0.2 ml of 5 mg/l) were extracted at basic pH (4 ml of 0.5 mol/l sodium hydroxide into *n*-heptane–isobutanol (96:4, v/v) and then back-extracted in 4 ml of 1 mol/l sulfuric acid. The solution was made basic with sodium hydroxide, the drugs were extracted into diethyl ether and the ether was evaporated. The residue was dissolved in 50 μl of absolute ethanol and 15 μl were injected for HPLC analysis. For GC, 1–2 μl was injected for analysis.

Peak height ratios of midazolam to that of flurazepam (the internal standard), were used to calculate the drug's concentration, by GC. For HPLC, peak height ratios of midazolam and 1-hydroxymethylmidazolam to that of flurazepam were determined at 254 and 220 nm.

RESULTS

Midazolam has a UV absorption maximum at 255 nm ($E_{1\%}^{1\text{cm}} = 650$) in an acidic pH. A shoulder is observed at 220 nm. HPLC chromatograms at 254 nm of an unextracted standard mixture, and serum extracts of standards of 250, 500, and 750 ng/ml are given in Fig. 1. Similar results were observed at 220 nm. Blank sera assayed by this procedure showed no significant peaks that might interfere with the analysis (Fig. 2). Retention times for the 1-hydroxymethyl metabolite and midazolam are 0.73 and 0.86 relative to flurazepam, which elutes in 13 min. Fig. 2 illustrates the use of this procedure to analyze sera of patients who were induced for anesthesia with midazolam. When peak heights of sera containing 250, 500 and 750 ng/ml of midazolam were plotted against concentration, the resulting line had a slope of 0.0031, a y-intercept of -0.02 , a standard error of estimate (S_{yx}) of 0.02 and a correlation coefficient of 0.99. Within-run absolute analytical recovery determined with serum extracts (0.25–0.75 mg/l) averaged $78 \pm 5\%$ ($n = 5$). Relative within-run

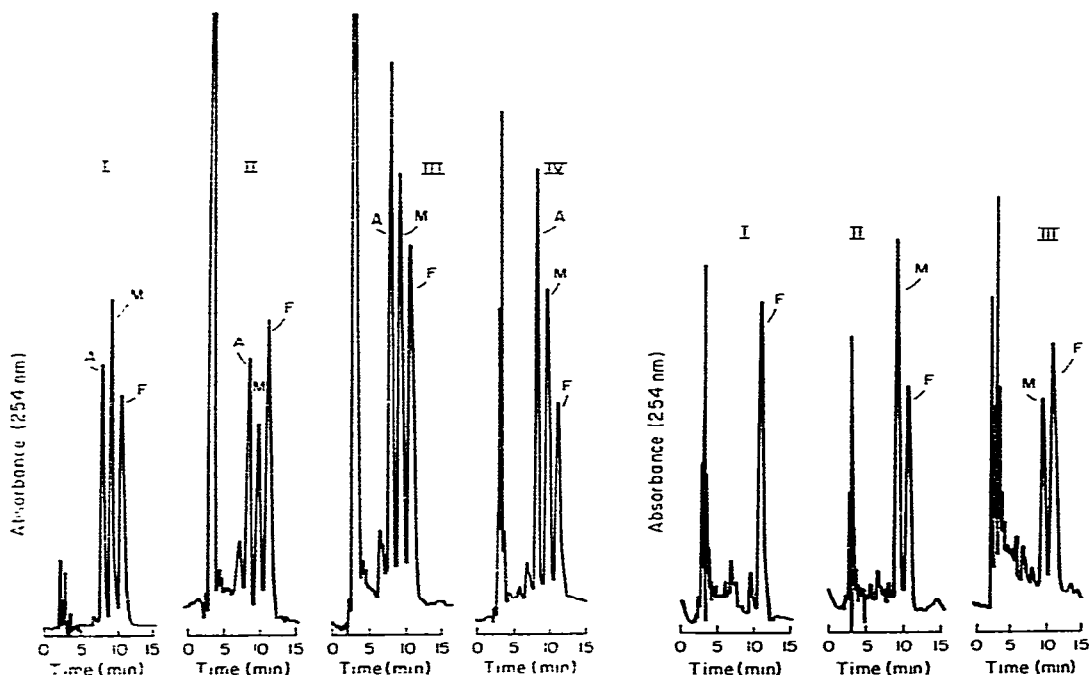


Fig. 1. Liquid chromatograms of (I) a mixture of pure unextracted standards 0.1 g/l, 1 μ l injected; and serum standards containing II, 250; III, 500 and IV, 750 ng/ml of midazolam and its 1-hydroxymethyl metabolite. Peaks: A = 1-hydroxymethylmidazolam; M = midazolam; F = flurazepam, internal standard, 450 ng/ml. Eluting solvent methanol-water (60:40, v/v) with 5 mmol/l octanesulfonic acid, pH adjusted to 3.5. Absorbance scale is variable from 0.005 to 0.02 units.

Fig. 2. Liquid chromatograms of patients' sera extracts taken at times 0 (I), 1–2 (II), and 13–14 (III) min after the administration of midazolam for induction of anesthesia. Concentration of midazolam (M) is 676 and 320 μ g/l in II and III respectively. F = Flurazepam (internal standard), 450 ng/ml. Notice the absence of 1-hydroxymethylmidazolam.

percentage recovery with serum extracts (0.25–0.75 mg/l) averaged $100 \pm 11\%$ ($n = 5$).

Within-run precisions (coefficients of variation) of serum-based controls containing 200 and 400 ng/ml of midazolam were 3 ($n = 4$) and 5% ($n = 3$), respectively. Between-run precision of a 500 ng/ml serum based standard was 10% ($n = 5$).

When peak heights of sera containing 250, 500, and 750 ng/ml of 1-hydroxymethylmidazolam were plotted against concentration, the resulting line had a slope of 0.0025, y-intercept of -0.02 , S_{yx} of 0.09, and a correlation coefficient of 0.99. The absolute recovery using serum standards (0.25–0.75 mg/l) averaged $97 \pm 5\%$ ($n = 5$) while the relative percent recovery averaged $100 \pm 5\%$ ($n = 5$). Within-run precision of serum-based controls containing 200 and 400 ng/ml of the 1-hydroxymethyl metabolite was 4% ($n = 4$ in each). Between-run precision of a 500 ng/ml serum standard was 11% ($n = 9$). Peak height ratios of sera extracts containing midazolam and 1-hydroxymethylmidazolam were linearly related to concentration up to 2 $\mu\text{g/ml}$. Diazepam interferes with midazolam while nordiazepam interferes with the 1-hydroxymethyl metabolite.

DISCUSSION

Midazolam maleate is an investigational benzodiazepine which is pharmacologically similar to diazepam. Its half-life, which is less than 3 h, is much shorter than the half-life of diazepam, which is greater than 20 h. Midazolam is, therefore, better suited for induction of anesthesia. Therapeutic and toxic ranges for midazolam have not yet been established for humans. Because 1-hydroxymethylmidazolam is the major reported metabolite of midazolam and since it does not chromatograph well by GC, we chose HPLC as an alternative means for performing patient serum determinations for midazolam and 1-hydroxymethylmidazolam. Our interest was to set up an assay for the determination of midazolam and its 1-hydroxymethyl metabolite, and then to determine parent drug and metabolite concentrations in patients' sera.

Parent drug and metabolite determinations are readily performed by HPLC as shown by Figs. 1 and 2. The same analysis performed by GC would require the formation of a derivative of 1-hydroxymethylmidazolam and would, therefore, be more complex than the present HPLC method. The relative within- and between-run recovery using serum standards averaged 100% for midazolam and 1-hydroxymethylmidazolam. In the case of the 1-hydroxymethyl metabolite the absolute recovery was close to 100%, which indicates that the 1-hydroxy metabolite is extracted better with the present procedure than midazolam. The absolute recovery of midazolam averaged 80%. Within-run precision for midazolam and 1-hydroxymethylmidazolam averaged 4% in the 250–750 ng/ml range by HPLC. In contrast the within-run precision for midazolam by GC in the 3 and 5 mg/l range was 6% [1]. The precision of the present method is 5% at serum midazolam concentrations of less than 50 ng/ml. This was established by running a 25 ng/ml serum standard in triplicate. It was not possible to obtain as good a sensitivity with the previously reported GC procedure [1] because of the high chromatographic background which was

observed at low concentrations in the vicinity of the midazolam peak. However, the previously reported GC procedure [1] gives results comparable to the present HPLC method at serum midazolam concentrations of greater than 100 ng/ml. Twenty-three serum samples analyzed first by GC and then HPLC gave a slope of 1.09, y -intercept of 120, a standard error of estimate (S_{yx}) of 111, and a correlation coefficient of 0.98.

The use of flurazepam as internal standard is important in order to retain the flexibility of performing the chromatographic analysis by GC or HPLC. If one is interested in performing analysis only by HPLC, prazepam, which has a retention time of 1.5 relative to flurazepam, is a preferred internal standard. Prazepam could not be used as an internal standard for GC since it interferes with midazolam [1]. Of the commonly prescribed drugs diazepam interferes with midazolam while nordiazepam, quinidine, methaqualone and oxazepam interfere with the 1-hydroxymethyl metabolite. The following drugs did not interfere: amitriptyline, nortriptyline, doxepin, imipramine, desipramine, procainamide, loxapine, phenobarbital, secobarbital, salicylate, phenytoin, meprobamate, glutethimide, and disopyramide. A check for the presence of interfering substances with HPLC is to monitor the peaks at two wavelengths. A comparison of patient's midazolam determinations at 254 and 220 nm is given in Fig. 3. Least squares analysis gave a slope of 1.09, y -intercept of -46, a standard error of estimate (S_{yx}) of 67 and a correlation coefficient of 0.99. The above results confirm that no detectable interference was observed with these sera midazolam determinations. In the case of an interfering substance such as diazepam the analysis could be performed by GC or GC-mass spectrometry as we have previously suggested [1, 2].

Patient samples analyzed for the presence of midazolam and 1-hydroxymethylmidazolam after dosing are given in Fig. 2. As can be seen, no detectable quantity of free 1-hydroxymethylmidazolam is observed in serum. Timed serum samples taken up to 8 h after dosing did not reveal the presence of the 1-hydroxymethyl metabolite. Serum midazolam concentrations decrease

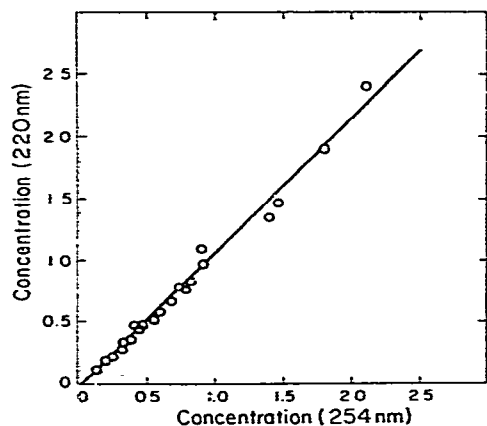


Fig. 3. Comparison of patients' sera midazolam determinations at 254 and 220 nm. See text for statistical analysis. Concentration in mg/l.

rapidly due to the distribution of the drug from the main (vascular) compartment into the peripheral (tissue) compartment. The half-life for the initial distribution (alpha-phase) from the present data is less than 10 min, with a half-life for the beta phase being 2–3 h. Thus at the end of 8 h, one would expect to see the presence of free 1-hydroxymetabolite if it is present in appreciable concentration in serum. A complete analysis of patient-results as well as the possible presence of other metabolites, such as desmethyl midazolam, is currently in progress. The present HPLC method is a sensitive direct procedure for the determination of midazolam and 1-hydroxymethylmidazolam. Analysis can be performed with a sensitivity of 15 ng/ml and a precision of less than 5%.

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